PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/70, 1/21, C07H 21/04
C07K 13/00, C12P 21/02

A1

(11) International Publication Number: WO 92/13955

(43) International Publication Date: 20 August 1992 (20.08.92)

(21) International Application Number: PCT/US92/00944

(22) International Filing Date: 6 February 1992 (06.02.92)

(30) Priority data:
652,531 6 February 1991 (06.02.91) US
745,382 14 August 1991 (14.08.91) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: PEPTIDE AND PROTEIN FUSIONS TO THIOREDOXIN AND THIOREDOXIN-LIKE MOLECULES

(57) Abstract

This invention provides a fusion molecule comprising a DNA sequence encoding a thioredoxin-like protein fused to the DNA sequence encoding a selected heterologous peptide or protein. The peptide or protein may be fused to the amino terminus of the thioredoxin-like molecule, the carboxyl terminus of the thioredoxin-like molecule, or within the thioredoxin-like molecule, for example at the active-site loop of said molecule. Expression of this fusion molecule under the control of a regulatory sequence capable of directing its expression in a desired host cell, produces high levels of stable and soluble fusion protein. The fusion protein, located in the bacterial cytoplasm, may be selectively released from the cell by osmotic shock or freeze/thaw procedures. It may be optionally cleaved to liberate the soluble, correctly folded heterologous protein from the thioredoxin-like portion.

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PEPTIDE AND PROTEIN FUSIONS TO THIOREDOXIN AND THIOREDOXIN-LIKE MOLECULES

The invention relates generally to the production of fusion proteins in prokaryotic and eukaryotic cells. More specifically, the invention relates to the expression in host cells of recombinant fusion sequences comprising thioredoxin or thioredoxin-like sequences fused to sequences for selected heterologous peptides or proteins, and the use of such fusion molecules to increase the production, activity, stability or solubility of recombinant proteins and peptides.

Background of the Invention

Many peptides and proteins can be produced via recombinant means in a variety of expression systems, e.g., various strains of bacterial, fungal, mammalian or insect cells. However, when bacteria are used as host cells for heterologous gene expression, several problems frequently occur.

For example, heterologous genes encoding small peptides are often poorly expressed in bacteria. Because of their size, most small peptides are unable to adopt stable, soluble conformations and are subject to intracellular degradation by proteases and peptidases present in the host cell. Those small peptides which do manage to accumulate when directly expressed in E. coli or other bacterial hosts are usually found in the insoluble or "inclusion body" fraction, an occurrence which renders them almost useless for screening purposes in biological or biochemical assays.

Moreover, even if small peptides are not produced in inclusion bodies, the production of small peptides by recombinant means as candidates for new drugs or enzyme inhibitors encounters further problems. Even small linear peptides can adopt an

enormous number of potential structures due to their degrees of conformational freedom. Thus a small peptide can have the 'desired' amino-acid sequence and yet have very low activity in an assay because the 'active' peptide conformation is only one of the many alternative structures adopted in free solution. This presents another difficulty encountered in producing small heterologous peptides recombinantly for effective research and therapeutic use.

Inclusion body formation is also frequently observed when the genes for heterologous proteins are expressed in bacterial cells. These inclusion bodies usually require further manipulations in order to solubilize and refold the heterologous protein, with conditions determined empirically and with uncertainty in each case.

If these additional procedures are not successful, little to no protein retaining bioactivity can be recovered from the host cells. Moreover, these additional processes are often technically difficult and prohibitively expensive for practical production of recombinant proteins for therapeutic, diagnostic or other research uses.

To overcome these problems, the art has employed certain peptides or proteins as fusion "partners" with a desired heterologous peptide or protein to enable the recombinant expression and/or secretion of small peptides or larger proteins as fusion proteins in bacterial expression systems. Among such fusion partners are included lacZ and trpE fusion proteins, maltose-binding protein fusions, and glutathione-S-transferase fusion proteins [See, generally, Current Protocols in Molecular Biology, Vol. 2, suppl. 10, publ. John Wiley and Sons, New York, NY, pp. 16.4.1-16.8.1 (1990); and Smith et al, Gene 67:31-40 (1988)]. U. S. Patent 4,801,536 describes the fusion of a bacterial flagellin protein to a desired protein to enable the production of a heterologous gene in a bacterial cell and its secr tion into the culture medium as a fusion protein. PCT Patent Publication WO91/11454 disclos s fusion proteins using

biotinylated renin as the fusion partner. The renin is immobilized on a purification column to facilitate separation and cleavage.

However, often fusions of desired peptides or proteins to other proteins (i.e., as fusion partners) at the amino- or carboxyl- termini of these fusion partner proteins have other potential disadvantages. Experience in E. coli has shown that a crucial factor in obtaining high levels of gene expression is the efficiency of translational initiation. Translational initiation in E. coli is very sensitive to the nucleotide sequence surrounding the initiating methionine codon of the desired heterologous peptide or protein sequence, although the rules governing this phenomenon are not clear. For this reason, fusions of sequences at the amino-terminus of many fusion partner proteins affects expression levels in an unpredictable manner. In addition there are numerous amino- and carboxy-peptidases in E. coli which degrade amino- or carboxyl-terminal peptide extensions to fusion partner proteins so that a number of the known fusion partners have a low success rate for producing stable fusion proteins.

The purification of proteins produced by recombinant expression systems is often a serious challenge. There is a continuing requirement for new and easier methods to produce homogeneous preparations of recombinant proteins, and yet a number of the fusion partners currently used in the art possess no inherent properties that would facilitate the purification process. Therefore, in the art of recombinant expression systems, there remains a need for new compositions and processes for the production and purification of stable, soluble peptides and proteins for use in research, diagnostic and therapeutic applications.

Summary of the Invention

In one aspect, the invention provides a fusion sequence comprising a thior doxin-like protein sequence fused to a

selected heterologous peptide or protein. The peptide or protein may be fused to the amino terminus of the thioredoxin-like sequence, the carboxyl terminus of the thioredoxin-like sequence, or within the thioredoxin-like sequence (e.g., within the active-site loop of thioredoxin). The fusion sequence according to this invention may optionally contain a linker peptide between the thioredoxin-like sequence and the selected peptide or protein. This linker provides, where needed, a selected cleavage site or a stretch of amino acids capable of preventing steric hindrance between the thioredoxin-like molecule and the selected peptide or protein.

As another aspect, the invention provides a DNA molecule encoding the fusion sequence defined above in association with, and under the control of, an expression control sequence capable of directing the expression of the fusion protein in a desired host cell.

Still a further aspect of the invention is a host cell transformed with, or having integrated into its genome, a DNA sequence comprising a thioredoxin-like DNA sequence fused to the DNA sequence of a selected heterologous peptide or protein. This fusion sequence is desirably under the control of an expression control sequence capable of directing the expression of a fusion protein in the cell.

As yet another aspect, there is provided a novel method for increasing the expression of soluble recombinant proteins. The method includes culturing under suitable conditions the above-described host cell to produce the fusion protein.

In one embodiment of this method, if the resulting fusion protein is cytoplasmic, the cell can be lysed by conventional means to obtain the soluble fusion protein. More preferably in the case of cytoplasmic fusion proteins, the method includes releasing the fusion protein from the host cell by applying osmotic shock or freeze/thaw treatments to the cell. In this case th fusion protein is sel ctively released from th interior f th cell via the z nes of adhesion that exist b tween th

inner and outer membranes of E. coli. The fusion protein is then purified by conventional means.

In another embodiment of the method, if a secretory leader is employed in the fusion protein construct, the fusion protein can be recovered from a periplasmic extract or from the cell culture medium.

An additional step in both of these methods is cleavage of the desired protein from the thioredoxin-like protein by conventional means.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

Summary of the Drawings

Fig. 1 illustrates the DNA sequence of the expression plasmid pALTRXA/EK/IL1$1_{\Delta}$ Pro-581 and the amino acid sequence for the fusion protein therein, described in Example 1.

Fig. 2 illustrates the DNA sequence and amino acid sequence of the macrophage inhibitory protein-1α (MIP-1α) protein used in the construction of a thioredoxin fusion protein described in Example 3.

Fig. 3 illustrates the DNA sequence and amino acid sequence of the bone morphogenetic protein-2 (BMP-2) protein used in the construction of a thioredoxin fusion protein described in Example 4.

Fig. 4 is a schematic drawing illustrating the insertion of an enterokinase cleavage site into the active-site loop of E. coli thioredoxin (trxA) described in Example 5.

Fig. 5 is a schematic drawing illustrating random peptide insertions into the active-site loop of E. coli thioredoxin (trxA) described in Example 5.

Fig. 6 illustrates the DNA sequence and amino acid sequence of the human interleukin-6 (IL-6) protein used in the construction of a thioredoxin fusi n pr tein described in Example 6.

Fig. 7 illustrates the DNA sequence and amino acid sequence of the M-CSF protein used in the construction of a thioredoxin fusion protein described in Example 7.

Detailed Description of the Invention

This invention permits the production of large amounts of heterologous peptides or proteins in a stable, soluble form in certain host cells that normally express limited amounts of such peptides or proteins. It enables release of the fusion protein from the production cells without the necessity of lysing the cells, thereby streamlining the purification process. Also, by using a small peptide insert in an internal region of the thioredoxin-like sequence (e.g. the active site loop of thioredoxin) the invention provides a ready cleavage site, accessible on the surface of the molecule. The fusion proteins of this invention also permit the desired peptide or protein to achieve its desired conformation.

According to the present invention, the DNA sequence encoding a heterologous peptide or protein selected for expression in a recombinant system is fused to a thioredoxin-like DNA sequence for expression in the host cell. A thioredoxin-like DNA sequence is defined herein as a DNA sequence encoding a protein or fragment of a protein characterized by an amino acid sequence having at least 18% homology with the amino acid sequence of E. coli thioredoxin over an amino acid sequence length of 80 amino acids. Alternatively, a thioredoxin DNA sequence is defined as a DNA sequence encoding a protein or fragment of a protein characterized by a crystalline structure substantially similar to that of human or E. coli thioredoxin. The DNA sequence of glutaredoxin is one such sequence. The amino acid sequence of E. coli thioredoxin is described in H. Eklund et al, EMBO J. 3:1443-1449 (1984). The three-dimensional structure of E. coli thioredoxin is depicted in Fig. 2 of A. Holmgren, J. Bi l. Chem. 264:13963-13966 (1989). Fig. 1 below nucleotid s 2242-2568 contains a DNA sequence encoding the E. coli

thioredoxin protein [Lim et al, J. Bacteriol. 163:311-316 (1985)]. The three latter publications are incorporated herein by reference for the purpose of providing information on thioredoxin which is known to one of skill in the art.

As the primary example of a thioredoxin-like protein useful in this invention, E. coli thioredoxin has the following characteristics. E. coli thioredoxin is a small protein, only 11.7 kD, and can be expressed to high levels (>10%, corresponding to a concentration of 15 uM if cells are lysed at 10 A_{550}/ml). The small size and capacity for high expression of the protein contributes to a high intracellular concentration. E. coli thioredoxin is further characterized by a very stable, tight structure which can minimize the effects on overall structural stability caused by fusion to the desired peptide or proteins.

The three dimensional structure of E. coli thioredoxin is known. It contains several surface loops, including a unique active site loop between residues Cys_{33} and Cys_{36} which protrudes from the body of the protein. This active site loop is an identifiable, accessible surface loop region and is not involved in any interactions with the rest of the protein that contribute to overall structural stability. It is therefore a good candidate as a site for peptide insertions. Both the amino- and carboxyl-termini of E. coli thioredoxin are on the surface of the protein, and are readily accessible for fusions.

E. coli thioredoxin is also stable to proteases. Thus, E. coli thioredoxin may be desirable for use in E. coli expression systems, because as an E. coli protein it is characterized by stability to E. coli proteases. E. coli thioredoxin is also stable to heat up to 80°C and to low pH. Other thioredoxin-like proteins encoded by thioredoxin-like DNA sequences useful in this invention may share the homologous amino acid sequences, and similar physical and structural characteristics. Thus, DNA sequences encoding other thioredoxin-like proteins may be used in place of E. coli thioredoxin according to this invention. For example, the DNA sequ nce encoding other species' thi redoxin,

e.g., human thioredoxin, may be employed in the compositions and methods of this invention. Both the primary sequence and computer-predicted secondary structures of human and E. coli thioredoxins are very similar. Human thioredoxin also carries the same active site loop as is found in the E. coli protein. Insertions into the human thioredoxin active site loop and on the amino and carboxyl termini may be as well tolerated as those in E. coli thioredoxin.

Other thioredoxin-like sequences which may be employed in this invention include all or portions of the proteins glutaredoxin and various species' homologs thereof [A. Holmgren, cited above]. Although E. coli glutaredoxin and E. coli thioredoxin share less than 20% amino acid homology, the two proteins do have conformational and functional similarities [Eklund et al, EMBO J. 3:1443-1449 (1984)].

All or a portion of the DNA sequence encoding protein disulfide isomerase (PDI) and various species' homologs thereof [J. E. Edman et al, Nature 317:267-270 (1985)] may also be employed as a thioredoxin-like DNA sequence, since a repeated domain of PDI shares >18% homology with E. coli thioredoxin. The two latter publications are incorporated herein by reference for the purpose of providing information on glutaredoxin and PDI which is known and available to one of skill in the art.

Similarly the DNA sequence encoding phosphoinositide-specific phospholipase C (PI-PLC), fragments thereof and various species' homologs thereof [C. F. Bennett et al, Nature 334:268-270 (1988)] may also be employed in the present invention as a thioredoxin-like sequence based on the amino acid sequence homology with E. coli thioredoxin. All or a portion of the DNA sequence encoding an endoplasmic reticulum protein, such as Erp72, or various species homologs thereof are also included as thioredoxin-like DNA sequences for the purposes of this invention [R. A. Mazzarella et al, J. Biol. Chem. 265:1094-1101 (1990)] based on amino acid sequence homology. Anoth r thioredoxin-like sequenc is a DNA sequenc which encodes all or a portion of an

adult T-cell leukemia-derived factor (ADF) or other species homologs thereof [N. Wakasugi et al, Proc. Natl. Acad. Sci., USA 87:8282-8286 (1990)] based on amino acid sequence homology to E. coli thioredoxin. The three latter publications are incorporated herein by reference for the purpose of providing information on PI-PLC, Erp72, and ADF which are known and available to one of skill in the art.

It is expected from the definition of thioredoxin-like DNA sequence used above that other sequences not specifically identified above, or perhaps not yet identified or published, may be useful as thioredoxin-like sequences based on their amino acid sequence similarities to E. coli thioredoxin and characteristic crystalline structural similarities to E. coli thioredoxin and the other thioredoxin-like proteins. Based on the above description, one of skill in the art should be able to select and identify, or, if desired, modify, a thioredoxin-like DNA sequence for use in this invention without resort to undue experimentation. For example, simple point mutations made to portions of native thioredoxin or native thioredoxin-like sequences which do not effect the structure of the resulting molecule are alternative thioredoxin-like sequences, as are allelic variants of native thioredoxin or native thioredoxin-like sequences.

DNA sequences which hybridize to the sequence for E. coli thioredoxin or its structural homologs under either stringent or relaxed hybridization also encode thioredoxin-like proteins for use in this invention. Stringent hybridization is defined herein as hybridization at 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for an hour. Alternatively stringent hybridization is defined as hybridization in 50% formamide, 4XSSC at 42°C. Non-stringent hybridization is defined herein as hybridizing in at are 4XSSC at 50°C, or hybridization with 30-40% formamide at 42°C. The use of all such thioredoxin-like sequences are beli ved to be encompassed in this invention.

C nstructi n f a fusi n s quenc f th present invention,

which comprises the DNA sequence of a selected peptide or protein and the DNA sequence of a thioredoxin-like sequence, employs conventional genetic engineering techniques [see, Sambrook et al, Molecular Cloning. A Laboratory Manual., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)]. Fusion sequences may be prepared in a number of different ways. For example, the selected heterologous protein may be fused to the amino terminus of the thioredoxin-like molecule. Alternatively, the selected protein sequence may be fused to the carboxyl terminus of the thioredoxin-like molecule. Small peptide sequences could also be fused to either of the above-mentioned positions of the thioredoxin-like sequence to produce them in a structurally unconstrained manner.

This fusion of a desired heterologous peptide or protein to the thioredoxin-like protein increases the stability of the peptide or protein. At either the amino or carboxyl terminus, the desired heterologous peptide or protein is fused in such a manner that the fusion does not destabilize the native structure of either protein. Additionally, fusion to the soluble thioredoxin-like protein improves the solubility of the selected heterologous peptide or protein.

It may be preferred for a variety of reasons that peptides be fused within the active site loop of the thioredoxin-like molecule. The face of thioredoxin surrounding the active site loop has evolved, in keeping with the protein's major function as a nonspecific protein disulfide oxido-reductase, to be able to interact with a wide variety of protein surfaces. The active site loop region is found between segments of strong secondary structure and offers many advantages for peptide fusions. A small peptide inserted into the active-site loop of a thioredoxin-like protein is present in a region of the protein which is not involved in maintaining tertiary structure. Therefore the structure of such a fusion protein should be stable. Previous work has shown that E. coli thioredoxin can be cleaved into two fragments at a position cl s to th active site

loop, and yet the tertiary interactions stabilizing the protein remain.

The active site loop of E. coli thioredoxin has the sequence NH_2...Cys_{33}-Gly-Pro-Cys_{36}...COOH. Fusing a selected peptide with a thioredoxin-like protein in the active loop portion of the protein constrains the peptide at both ends, reducing the degrees of conformational freedom of the peptide, and consequently reducing the number of alternative structures taken by the peptide. The inserted peptide is bound at each end by cysteine residues, which may form a disulfide linkage to each other as they do in native thioredoxin and further limit the conformational freedom of the inserted peptide.

Moreover, this invention places the peptide on the surface of the thioredoxin-like protein. Thus the invention provides a distinct advantage for use of the peptides in screening for bioactive peptide conformations and other assays by presenting peptides inserted in the active site loop in this structural context.

Additionally the fusion of a peptide into the loop protects it from the actions of E. coli amino- and carboxyl-peptidases. Further a restriction endonuclease cleavage site RsrII already exists in the portion of the E. coli thioredoxin DNA sequence encoding the loop region at precisely the correct position for a peptide fusion [see Figure 4]. RsrII recognizes the DNA sequence CGG(A/T)CCG leaving a three nucleotide long 5'-protruding sticky end. DNA bearing the complementary sticky ends will therefore insert at this site in just one orientation.

A fusion sequence of a thioredoxin-like sequence and a desired protein or peptide sequence according to this invention may optionally contain a linker peptide inserted between the thioredoxin-like sequence and the selected heterologous peptide or protein. This linker sequence may encode, if desired, a polypeptide which is selectably cleavable or digestible by conventional chemical or enzymatic methods. For example, the selected cleavage site may be an enzymatic cleavage site.

Examples of enzymatic cleavage sites include sites for cleavage by a proteolytic enzyme, such as enterokinase, Factor Xa, trypsin, collagenase, and thrombin. Alternatively, the cleavage site in the linker may be a site capable of being cleaved upon exposure to a selected chemical, e.g., cyanogen bromide, hydroxylamine, or low pH.

Cleavage at the selected cleavage site enables separation of the heterologous protein or peptide from the thioredoxin fusion protein to yield the mature heterologous peptide or protein. The mature peptide or protein may then be obtained in purified form, free from any polypeptide fragment of the thioredoxin-like protein to which it was previously linked. The cleavage site, if inserted into a linker useful in the fusion sequences of this invention, does not limit this invention. Any desired cleavage site, of which many are known in the art, may be used for this purpose.

The optional linker sequence of a fusion sequence of the present invention may serve a purpose other than the provision of a cleavage site. The linker may also be a simple amino acid sequence of a sufficient length to prevent any steric hindrance between the thioredoxin-like molecule and the selected heterologous peptide or protein.

Whether or not such a linker sequence is necessary will depend upon the structural characteristics of the selected heterologous peptide or protein and whether or not the resulting fusion protein is useful without cleavage. For example, where the thioredoxin-like sequence is a human sequence, the fusion protein may itself be useful as a therapeutic without cleavage of the selected protein or peptide therefrom. Alternatively, where the mature protein sequence may be naturally cleaved, no linker may be needed.

In one embodiment therefore, the fusion sequence of this invention contains a thioredoxin-like sequence fused directly at its amino or carboxyl terminal end to the s quenc f the selected p ptid r protein. The r sulting fusion prot in is

thus a soluble cytoplasmic fusion protein. In another embodiment, the fusion sequence further comprises a linker sequence interposed between the thioredoxin-like sequence and the selected peptide or protein sequence. This fusion protein is also produced as a soluble cytoplasmic protein. Similarly, where the selected peptide sequence is inserted into the active site loop region or elsewhere within the thioredoxin-like sequence, a cytoplasmic fusion protein is produced.

The cytoplasmic fusion protein can be purified by conventional means. Preferably, as a novel aspect of the present invention, several thioredoxin fusion proteins of this invention may be purified by exploiting an unusual property of thioredoxin. The cytoplasm of E. coli is effectively isolated from the external medium by a cell envelope comprising two membranes, inner and outer, separated from each other by a periplasmic space within which lies a rigid peptidoglycan cell wall. The peptidoglycan wall contributes both shape and strength to the cell. At certain locations in the cell envelope there are "gaps" (called variously Bayer patches, Bayer junctions or adhesion sites) in the peptidoglycan wall where the inner and outer membranes appear to meet and perhaps fuse together. See, M. E. Bayer, J. Bacteriol. 93:1104-1112 (1967) and J. Gen. Microbiol. 53:395-404 (1968). Most of the cellular thioredoxin lies loosely associated with the inner surface of the membrane at these adhesion sites and can be quantitatively expelled from the cell through these adhesion sites by a sudden osmotic shock or by a simple freeze/thaw procedure. See C. A. Lunn and V. P. Pigiet, J. Biol. Chem. 257:11424-11430 (1982) and in "Thioredoxin and Glutaredoxin Systems: Structure and Function:165-176 (1986) ed. A. Holmgren et al., Raven Press, New York. To a lesser extent some EF-Tu (elongation factor-Tu) can be expelled in the same way [Jacobson et al, Biochemistry 15:2297-2302 (1976)], but, with the exception of the periplasmic contents, the vast majority of E. coli proteins cannot be released by these treatments.

Although there have been reports of the release by osmotic

shock of a limited number of heterologous proteins produced in the cytoplasm of E. coli [Denefle et al, Gene 85:499-510 (1989); Joseph-Liauzun et al, Gene 86:291-295 (1990); Rosenwasser et al, J. Biol. Chem. 265:13066-13073 (1990)], the ability to be so released is a rare and desirable property not shared by the majority of heterologous proteins. Fusion of a heterologous protein to thioredoxin as described by the present invention not only enhances its expression, solubility and stability as described above, but may also provide for its release from the cell by osmotic shock or freeze/thaw treatments, greatly simplifying its purification. The thioredoxin portion of the fusion protein in some cases, e.g., with MIP, directs the fusion protein towards the adhesion sites, from where it can be released to the exterior by these treatments.

In another embodiment the present invention may employ another component, that is, a secretory leader sequence, among which many are known in the art, e.g. leader sequences of phoA, MBP, β-lactamase, operatively linked in frame to the fusion protein of this invention to enable the expression and secretion of the mature fusion protein into the bacterial periplasmic space or culture medium. This leader sequence may be fused to the amino terminus of the thioredoxin-like molecule when the selected peptide or protein sequence is fused to the carboxyl terminus or to an internal site within the thioredoxin-like sequence. An optional linker could also be present when the peptide or protein is fused at the carboxyl terminus. It is expected that this fusion sequence construct when expressed in an appropriate host cell would be expressed as a secreted fusion protein rather than a cytoplasmic fusion protein. However stability, solubility and high expression should characterize fusion proteins produced using any of these alternative embodiments.

This invention is not limited to any specific type of heterologous peptide or protein. A wide variety of heterologous genes or gen fragments are useful in forming th fusi n sequences of the present inventi n. While the comp sitions and

methods of this invention are most useful for peptides or proteins which are not expressed, expressed in inclusion bodies, or expressed in very small amounts in bacterial and yeast hosts, the heterologous peptides or proteins can include any peptide or protein useful for human or veterinary therapy, diagnostic or research applications in any expression system. For example, hormones, cytokines, growth or inhibitory factors, enzymes, modified or wholly synthetic proteins or peptides can be produced according to this invention in bacterial, yeast, mammalian or other eukaryotic cells and expression systems suitable therefor.

In the examples below illustrating this invention, the proteins expressed by this invention include IL-11, MIP-1α, IL-6, M-CSF, a bone inductive factor called BMP-2, and a variety of small peptides of random sequence. These proteins include examples of proteins which, when expressed without a thioredoxin fusion partner, are unstable in E. coli or are found in inclusion bodies.

A variety of DNA molecules incorporating the above-described fusion sequences may be constructed for expressing the heterologous peptide or protein according to this invention. At a minimum a desirable DNA sequence according to this invention comprises a fusion sequence described above, in association with, and under the control of, an expression control sequence capable of directing the expression of the fusion protein in a desired host cell. For example, where the host cell is an E. coli strain, the DNA molecule desirably contains a promoter which functions in E. coli, a ribosome binding site, and optionally, a selectable marker gene and an origin of replication if the DNA molecule is extra chromosomal. Numerous bacterial expression vectors containing these components are known in the art for bacterial expression, and can easily be constructed by standard molecular biology techniques. Similarly known yeast and mammalian cell vectors and vector components may be utilized where the host cell is a yeast cell or a mammalian cell.

The DNA molecules containing the fusion sequences may be

further modified to contain different codons to optimize expression in the selected host cell, as is known in the art.

These DNA molecules may additionally contain multiple copies of the thioredoxin-like DNA sequence, with the heterologous protein fused to only one of the DNA sequences, or with the heterologous protein fused to all copies of the thioredoxin-like sequence. It may also be possible to integrate a thioredoxin-like/heterologous peptide or protein-encoding fusion sequence into the chromosome of a selected host to either replace or duplicate a native thioredoxin-like sequence.

Host cells suitable for the present invention are preferably bacterial cells. For example, the various strains of E. coli (e.g., HB101, W3110 and strains used in the following examples) are well-known as host cells in the field of biotechnology. E. coli strain GI724, used in the following examples, has been deposited with a United States microorganism depository as described in detail below. Various strains of B. subtilis, Pseudomonas, and other bacteria may also be employed in this method.

Many strains of yeast and other eukaryotic cells known to those skilled in the art may also be useful as host cells for expression of the polypeptides of the present invention. Similarly known mammalian cells may also be employed in the expression of these fusion proteins.

To produce the fusion protein of this invention, the host cell is either transformed with, or has integrated into its genome, a DNA molecule comprising a thioredoxin-like DNA sequence fused to the DNA sequence of a selected heterologous peptide or protein, desirably under the control of an expression control sequence capable of directing the expression of a fusion protein. The host cell is then cultured under known conditions suitable for fusion protein production. If the fusion protein accumulates in the cytoplasm of the cell it may be released by conventional bacterial cell lysis t chniques and purifi d by c nventi nal procedures including s lectiv precipitati ns, s lubilizations

and column chromatographic methods. If a secretory leader is incorporated into the fusion molecule substantial purification is achieved when the fusion protein is secreted into the periplasmic space or the growth medium.

Alternatively, for cytoplasmic thioredoxin fusion proteins, a selective release from the cell may be achieved by osmotic shock or freeze/thaw procedures. Although final purification is still required for most purposes, the initial purity of fusion proteins in preparations resulting from these procedures is superior to that obtained in conventional whole cell lysates, reducing the number of subsequent purification steps required to attain homogeneity. In a typical osmotic shock procedure, the packed cells containing the fusion protein are resuspended on ice in a buffer containing EDTA and having a high osmolarity, usually due to the inclusion of a solute, such as 20% w/v sucrose, in the buffer which cannot readily cross the cytoplasmic membrane. During a brief incubation on ice the cells plasmolyze as water leaves the cytoplasm down the osmotic gradient. The cells are then switched into a buffer of low osmolarity, and during the osmotic re-equilibration both the contents of the periplasm and proteins localized at the Bayer patches are released to the exterior. A simple centrifugation following this release removes the majority of bacterial cell-derived contaminants from the fusion protein preparation. Alternatively, in a freeze/thaw procedure the packed cells containing the fusion protein are first resuspended in a buffer containing EDTA and are then frozen. Fusion protein release is subsequently achieved by allowing the frozen cell suspension to thaw. The majority of contaminants can be removed as described above by a centrifugation step. The fusion protein is further purified by well-known conventional methods.

These treatments typically release at least 30% of the fusion proteins without lysing the cell cultures. The success of these procedures in rel asing significant amounts f a wide variety of thi r doxin fusion proteins is surprising, since such

techniques are not generally successful with a wide range of proteins. The ability of these fusion proteins to be substantially purified by such treatments, which are significantly simpler and less expensive than the purification methods required by other fusion protein systems, may provide the fusion proteins of the invention with a significant advantage over other systems which are used to produce proteins in E. coli.

The resulting fusion protein is stable and soluble, often with the heterologous peptide or protein retaining its bioactivity. The heterologous peptide or protein may optionally be separated from the thioredoxin-like protein by cleavage, as discussed above.

In the specific and illustrative embodiments of the compositions and methods of this invention, the E. coli thioredoxin (trxA) gene has been cloned and placed in an E. coli expression system. An expression plasmid pALtrxA-781 was constructed. This plasmid containing modified IL-11 fused to the thioredoxin sequence and called pALtrxA/EK/IL11$_{\Delta}$Pro-581 is described below in Example 1 and in Fig. 1. A modified version of this plasmid containing a different ribosome binding site was employed in the other examples and is specifically described in Example 3. Other conventional vectors may be employed in this invention. The invention is not limited to the plasmids described in these examples.

Plasmid pALtrxA-781 (without the modified IL-11) directs the accumulation of >10% of the total cell protein as thioredoxin in E. coli host strain GI724. Examples 2 through 6 describe the use of this plasmid to form and express thioredoxin fusion proteins with BMP-2, IL-6 and MIP-1α, which are polypeptides.

As an example of the expression of small peptides inserted into the active-site loop, a derivative of pALtrxA-781 has been constructed in which a 13 amino-acid linker peptide sequence containing a cleavage site for the specific protease enterokinase [Leipnieks and Light, J. Biol. Chem. 254:1077-1083 (1979)] has been fused into the active site loop of thioredoxin. This

plasmid (pALtrxA-EK) directs the accumulation of >10% of the total cell protein as the fusion protein. The fusion protein is all soluble, indicating that it has probably adopted a 'native' tertiary structure. It is equally as stable as wild type thioredoxin to prolonged incubations at 80°C, suggesting that the strong tertiary structure of thioredoxin has not been compromised by the insertion into the active site loop. The fusion protein is specifically cleaved by enterokinase, whereas thioredoxin is not, indicating that the peptide inserted into the active site loop is present on the surface of the fusion protein.

As described in more detail in Example 5 below, fusions of small peptides were made into the active site loop of thioredoxin. The inserted peptides were 14 residues long and were of totally random composition to test the ability of the system to deal with hydrophobic, hydrophilic and neutral sequences.

The methods and compositions of this invention permit the production of proteins and peptides useful in research, diagnostic and therapeutic fields. The production of fusion proteins according to this invention has a number of advantages. As one example, the production of a selected protein by the present invention as a carboxyl-terminal fusion to E. coli thioredoxin, or another thioredoxin-like protein, enables avoidance of translation initiation problems often encountered in the production of eukaryotic proteins in E. coli. Additionally the initiator methionine usually remaining on the amino-terminus of the heterologous protein is not present and does not have to be removed when the heterologous protein is made as a carboxyl terminal thioredoxin fusion.

The production of fusion proteins according to this invention reliably improves solubility of desired heterologous proteins and enhances their stability to proteases in the expression system. This invention also enables high level xpr ssi n f certain desirable th rapeutic prot ins, e.g., IL-11, which are otherwise produced at low levels in bact rial host

cells.

This invention may also confer heat stability to the fusion protein, especially if the heterologous protein itself is heat stable. Because thioredoxin, and presumably all thioredoxin-like proteins, are heat stable up to 80°C, the present invention may enable the use of a simple heat treatment as an initial effective purification step for some thioredoxin fusion proteins.

In addition to providing high levels of the selected heterologous proteins or peptides upon cleavage from the fusion protein for therapeutic or other uses, the fusion proteins or fusion peptides of the present invention may themselves be useful as therapeutics. Further the thioredoxin-like fusion proteins may provide a vehicle for the delivery of bioactive peptides. As one example, human thioredoxin would not be antigenic in humans, and therefore a fusion protein of the present invention with human thioredoxin may be useful as a vehicle for delivering to humans the biologically active peptide to which it is fused. Because human thioredoxin is an intracellular protein, human thioredoxin fusion proteins may be produced in an E. coli intracellular expression system. Thus this invention also provides a method for delivering biologically active peptides or proteins to a patient in the form of a fusion protein with an acceptable thioredoxin-like protein.

The present invention also provides methods and reagents for screening libraries of random peptides for their potential enzyme inhibitory, hormone/growth factor agonist and hormone/growth factor antagonist activity. Also provided are methods and reagents for the mapping of known protein sequences for regions of potential interest, including receptor binding sites, substrate binding sites, phosphorylation/modification sites, protease cleavage sites, and epitopes.

Bacterial colonies expressing thioredoxin-like/random peptide fusion proteins may be screened using radiolabelled proteins such as hormones or growth factors as probes. Positives arising from this type of screen would identify mimics of

receptor binding sites and may lead to the design of compounds with therapeutic uses. Bacterial colonies expressing thioredoxin-like random peptide fusion proteins may also be screened using antibodies raised against native, active hormones or growth factors. Positives arising from this type of screen could be mimics of surface epitopes present on the original antigen. Where such surface epitopes are responsible for receptor binding, the 'positive' fusion proteins would have biological activity.

Additionally, the thioredoxin-like fusion proteins or fusion peptides of this invention may also be employed to develop monoclonal and polyclonal antibodies, or recombinant antibodies or chimeric antibodies, generated by known methods for diagnostic, purification or therapeutic use. Studies of thioredoxin-like molecules indicate a possible B cell/T cell growth factor activity [N. Wakasuki et al, cited above], which may enhance immune response. The fusion proteins or peptides of the present invention may be employed as antigens to elicit desirable antibodies, which themselves may be further manipulated by known techniques into monoclonal or recombinant antibodies.

Alternatively, antibodies elicited to thioredoxin-like sequences may also be useful in the purification of many different thioredoxin fusion proteins.

The following examples illustrate embodiments of the present invention, but are not intended to limit the scope of the disclosure.

EXAMPLE 1 - THIOREDOXIN-IL-11 FUSION MOLECULE

A thioredoxin-like fusion molecule of the present invention was prepared using E. coli thioredoxin as the thioredoxin-like sequence and recombinant IL-11 as the selected heterologous protein. The DNA and amino acid sequence of IL-11 has been published. See Paul et al, Proc. Natl. Acad. Sci. U.S.A. 87:7512-7516 (1990) and PCT Patent publication WO91/0749, publish d May 30, 1991. IL-11 DNA can b obtain d by cloning

based on its published sequence. The E. coli thioredoxin (trxA) gene was cloned based on its published sequence and employed to construct various related E. coli expression plasmids using standard DNA manipulation techniques, described extensively by Sambrook, Fritsch and Maniatis, Molecular Cloning. A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

A first expression plasmid pALTRxa-781 was constructed containing the E. coli trxA gene without fusion to another sequence. This plasmid further contained sequences which are described in detail below for the related IL-11 fusion plasmid. This first plasmid, which directs the accumulation of >10% of the total cell protein as thioredoxin in an E. coli host strain GI724, was further manipulated as described below for the construction of a trxA/IL-11 fusion sequence.

The entire sequence of the related plasmid expression vector, pALtrxA/EK/IL11$_{\Delta}$Pro-581, is illustrated in Fig. 1 and contains the following principal features:

Nucleotides 1-2060 contain DNA sequences originating from the plasmid pUC-18 [Norrander et al, Gene 26: 101-106 (1983)] including sequences containing the gene for β-lactamase which confers resistance to the antibiotic ampicillin in host E. coli strains, and a colE1-derived origin of replication. Nucleotides 2061-2221 contain DNA sequences for the major leftward promoter (pL) of bacteriophage λ [Sanger et al, J. Mol. Biol. 162:729-773 (1982)], including three operator sequences, O_L1, O_L2 and O_L3. The operators are the binding sites for λ cI repressor protein, intracellular levels of which control the amount of transcription initiation from pL. Nucleotides 2222-2241 contain a strong ribosome binding sequence derived from that of gene 10 of bacteriophage T7 [Dunn and Studier J. Mol. Biol. 166:477-535 (1983)].

Nucleotides 2242-2568 contain a DNA sequence encoding the E. coli thioredoxin protein [Lim et al, J. Bacteriol. 163:311-316 (1985)]. Ther is no translati n termination codon at the end of

the thioredoxin coding sequence in this plasmid.

Nucleotides 2569-2583 contain DNA sequence encoding the amino acid sequence for a short, hydrophilic, flexible spacer peptide "--GSGSG--". Nucleotides 2584-2598 provide DNA sequence encoding the amino acid sequence for the cleavage recognition site of enterokinase (EC 3.4.4.8), "--DDDDK--" [Maroux et al, J. Biol. Chem. 246:5031-5039 (1971)].

Nucleotides 2599-3132 contain DNA sequence encoding the amino acid sequence of a modified form of mature human IL-11 [Paul et al, Proc. Natl. Acad. Sci. USA 87:7512-7516 (1990)], deleted for the N-terminal prolyl-residue normally found in the natural protein. The sequence includes a translation termination codon at the 3'-end of the IL-11 sequence.

Nucleotides 3133-3159 provide a "Linker" DNA sequence containing restriction endonuclease sites. Nucleotides 3160-3232 provide a transcription termination sequence based on that of the E. coli aspA gene [Takagi et al, Nucl. Acids Res. 13:2063-2074 (1985)]. Nucleotides 3233-3632 are DNA sequences derived from pUC-18.

As described in Example 2 below, when cultured under the appropriate conditions in a suitable E. coli host strain, this plasmid vector can direct the production of high levels (approximately 10% of the total cellular protein) of a thioredoxin-IL-11 fusion protein. By contrast, when not fused to thioredoxin, IL-11 accumulated to only 0.2% of the total cellular protein when expressed in an analogous host/vector system.

EXAMPLE 2 - EXPRESSION OF A FUSION PROTEIN

A thioredoxin-IL-11 fusion protein was produced according to the following protocol using the plasmid constructed as described in Example 1. pALtrxA/EK/IL11$_{\Delta}$Pro-581 was transformed into the E. coli host strain GI724 (F^-, $lacI^q$, $lacP^{L8}$, ampC::λ cI^+) by the procedure of Dagert and Ehrlich, Gene 6:23 (1979). The untransformed host strain E. coli GI724 was deposit d with the American Type Culture Collection, 12301 Parklawn Driv ,

Rockville, Maryland on January 31, 1991 under ATCC No. 55151 for patent purposes pursuant to applicable laws and regulations. Transformants were selected on 1.5% w/v agar plates containing IMC medium, which is composed of M9 medium [Miller, "Experiments in Molecular Genetics", Cold Spring Harbor Laboratory, New York (1972)] supplemented with 0.5% w/v glucose, 0.2% w/v casamino acids and 100 µg/ml ampicillin.

GI724 contains a copy of the wild-type λ cI repressor gene stably integrated into the chromosome at the ampC locus, where it has been placed under the transcriptional control of Salmonella typhimurium trp promoter/operator sequences. In GI724, λ cI protein is made only during growth in tryptophan-free media, such as minimal media or a minimal medium supplemented with casamino acids such as IMC, described above. Addition of tryptophan to a culture of GI724 will repress the trp promoter and turn off synthesis of λ cI, gradually causing the induction of transcription from pL promoters if they are present in the cell.

GI724 transformed with pALtrxA/EK/IL11Δ Pro-581 was grown at 37°C to an A_{550} of 0.5 in IMC medium. Tryptophan was added to a final concentration of 100 µg/ml and the culture incubated for a further 4 hours. During this time thioredoxin-IL-11 fusion protein accumulated to approximately 10% of the total cell protein.

All of the fusion protein was found to be in the soluble cellular fraction, and was purified as follows. Cells were lysed in a french pressure cell at 20,000 psi in 50 mM HEPES pH 8.0, 1 mM phenylmethylsulfonyl fluoride. The lysate was clarified by centrifugation at 15,000 x g for 30 minutes and the supernatant loaded onto a QAE-Toyopearl column. The flow-through fractions were discarded and the fusion protein eluted with 50 mM HEPES pH 8.0, 100 mM NaCl. The eluate was adjusted to 2M NaCl and loaded onto a column of phenyl-Toyopearl. The flow-through fractions were again discarded and the fusion protein eluted with 50 mM HEPES pH 8.0, 0.5 M NaCl.

Th fusi n protein was then dialyzed against 25 mM HEPES pH

8.0 and was >80% pure at this stage. By T1165 bioassay [Paul et al, cited above] the purified thioredoxin-IL-11 protein exhibited an activity of $8x10^5$U/mg. This value agrees closely on a molar basis with the activity of $2x10^6$U/mg found for COS cell-derived IL-11 purified to homogeneity and measured for activity in the same assay. One milligram of the fusion protein was then cleaved at 37°C for 20 hours with 1000 units of bovine enterokinase [Leipnieks and Light, J. Biol. Chem. 254:1677-1683 (1979)] in 1 ml 10mM Tris-Cl (pH 8.0)/10mM $CaCl_2$. IL-11 was recovered from the reaction products by passing them over a QAE-Toyopearl column in 25 mM HEPES pH 8.0, where homogeneous IL-11 was found in the flow-through fractions. Uncleaved fusion protein, thioredoxin and enterokinase remained bound on the column.

The homogeneous IL-11 prepared in this manner had a bioactivity in the T1165 assay of $2.5x10^6$ U/mg. Its physical and chemical properties were determined as follows:

(1) Molecular Weight

The molecular weight of the IL-11 was found to be about 21 kD as measured by 10% SDS-PAGE under reducing conditions (tricine system) in accordance with the methods of Schagger, et al., Anal Biochem. 166:368-379. (1987). The compound ran as a single band.

(2) Endotoxin Content

The endotoxin content of the IL-11 was found to be less than 0.1 nanogram per milligram IL-11 in the LAL (Limulus amebocyte lysate, Pyrotel, available from Associates of Cape Cod, Inc., Woods Hole, Massachusetts, U.S.A.) assay, conducted in accordance with the manufacturer's instructions.

(3) Isoelectric Point

The theoretical isoelectric point of IL-11 is pH 11.70. As measured by polyacrylamide gel isoelectric focusing using an LKB Ampholine PAGplate with a pH range from 3.5 to 9.5, the IL-11 ran at greater than 9.5. An exact measurement could not b taken

because IL-11 is too basic a protein for the reliable gels available.

(4) Fluorescence Absorption Spectrum

Fluorescence absorption spectrum of the IL-11, as measured on a 0.1% aqueous solution in a 1 cm quartz cell showed an emission maximum at 335-337 nm.

(5) UV Absorption

UV absorption of the IL-11 on a 0.1% aqueous solution in a 1 cm quartz cell showed an absorbance maximum at 278-280 nm.

(6) Amino Acid Composition

The theoretical amino acid composition for IL-11, based on its amino acid sequence is as follow:

Amino Acid	Number	Mole %
Ala	20	11.3
Asp Acid	11	6.22
Cysteine	0	
Glu	3	1.70
Phe	1	0.57
Gly	14	7.91
His	4	2.26
Ile	2	1.13
Lys	3	1.70
Leu	41	23.16
Met	2	1.13
Asn	1	0.57
Pro	21	11.86
Gln	7	3.96
Arg	18	10.17
Ser	11	6.22
Thr	9	5.09
Val	5	2.83
Trp	3	1.70
Tyr	1	0.57

A sample of homogenous IL-11 was subjected to vapor phase hydrolysis as follows:

6 N HCl and 2 N Ph nol reagent wer added to hydrolysis vessel in which tubes containing 45 µl f 1:10 dilut d (w/$H_2$0)

IL-11, concentrated to dryness are inserted. Samples were sealed under vacuum and hydrolyzed for 36 hours at 110°C. After the hydrolysis, samples were dried and resuspended in 500 µl Na-S sample dilution buffer. Amino acid analysis was performed on a Beckman 7300 automated amino acid analyzer. A cation exchange column was used for separation of amino acids following post column derivatization with ninhydrin. Primary amino acids were detected at 570 nm and secondary amino acids were detected at 440 nm. Eight point calibration curves were constructed for each of the amino acids.

Because certain amino acids are typically not recovered, results for only 5 amino acids are given below. Since the hydrolysis was done without desalting the protein, 100% recovery was achieved for most of the amino acids.

The relative recovery of each individual amino acid residue per molecule of recombinant IL-11 was determined by normalizing GLX = 10 (the predicted number of glutamine and glutamic acid residue in IL-11 based on cDNA sequence). The value obtained for the recovery of GLX in picomoles was divided by 10 to obtain the GLX quotient. Dividing the value obtained for the recovery in picomoles of each amino acid by the GLX quotient for that sample gives a number that represents the relative recovery of each amino acid in the sample, normalized to the quantitative recovery of GLX residues. The correlation coefficient comparing the expected versus the average number of residues of each amino acid observed is greater than 0.985, indicating that the number of residues observed for each amino acid is in good agreement with that predicted sequence.

0	Amino Acids	1 No. of Residues Calculated	2 No. of Residues Expected	3 Correlation Coefficient
1	Asp	12.78	12	
2	Glu	10.00	10	
3	Gly	12.80	14	0.9852
4	Arg	16.10	18	
5	Pro	18.40	21	

(7) Amino Terminus Sequencing

IL-11 (buffered in 95% acetonitrile TFA) was sequenced using an ABI 471A protein sequencer (ABI, Inc.) in accordance with the manufacturer's instructions. Amino terminus sequencing confirmed that the thioredoxin fusion protein produced IL-11 contained the correct IL-11 amino acid sequence and only one amino terminus observed.

(8) Peptide Mapping

The IL-11 was cleaved with Endoproteinase Asp-N (Boehringer Mannheim) (1:500 ratio of Asp-N to IL-11) in 10 mM Tris, pH 8, 1 M urea and 2 mM 4-aminobenzamidine dihydrochloride (PABA), at 37°C for 4 hours. The sample was then run on HPLC on a C4 Vydac column using an A buffer of 50 mM $NaHPO_4$, pH 4.3, in dH_2O, a B buffer of 100% isopropanol with a gradient at 1 ml/min from 100%A to 25%A and 75%B (changing 1%/minute). The eluted peptide fragments were then sequenced using an ABI 471A protein sequencer (ABI, Inc.) in accordance with the manufacturer's instructions. Peptide mapped confirmed the IL-11 produced from the thioredoxin fusion protein contained the proper IL-11 N-terminal and C-terminal sequences.

(9) Solubility

IL-11 protein was tested for solubility in the substances below with the following results:

Water	very soluble
Ethyl Alcohol	very soluble
Acetone	very soluble
1M sodium chloride	very soluble
10% sucrose	very soluble

(10) Sugar Composition and Protein/Polysaccharide Content in %

The absence of sugar moieties attached to the polypeptide backbone of the IL-11 protein is indicated by its amino acid sequenc , which contains none f the typical sugar attachment sit s.

EXAMPLE 3 - THIOREDOXIN-MIP FUSION MOLECULE

Human macrophage inflammatory protein 1α (MIP-1α) was expressed at high levels in E. coli as a thioredoxin fusion protein using an expression vector similar to pALtrxA/EK/IL11Δ Pro-581 described in Example 1 above but modified in the following manner to replace the ribosome binding site of bacteriophage T7 with that of λ CII. In the plasmid of Example 1, nucleotides 2222 and 2241 were removed by conventional means. Inserted in place of those nucleotides was a sequence of nucleotides formed by nucleotides 35566 to 35472 and 38137 to 38361 from bacteriophage lambda as described in Sanger et al (1982) cited above. This reference is incorporated by reference for the purpose of disclosing this sequence. To express a thioredoxin-MIP-1α fusion the DNA sequence in the thusly-modified pALtrxA/EK/IL11Δ Pro-581 encoding human IL-11 (nucleotides 2599-3132) is replaced by the 213 nucleotide DNA sequence shown in Fig. 2 encoding full-length, mature human MIP-1α [Nakao et al, Mol. Cell. Biol. 10:3646-3658 (1990)].

The host strain and expression protocol used for the production of thioredoxin-MIP-1α fusion protein are as described in Example 1. As was seen with the thioredoxin-IL-11 fusion protein, all of the thioredoxin-MIP-1α fusion protein was found in the soluble cellular fraction, representing up to 20% of the total protein.

Cells were lysed as in Example 1 to give a protein concentration in the crude lysate of 10 mg/ml. This lysate was then heated at 80°C for 10 min to precipitate the majority of contaminating E. coli proteins and was clarified by centrifugation at 130,000 x g for 60 minutes. The pellet was discarded and the supernatant loaded onto a Mono Q column. The fusion protein eluted at approximately 0.5 M NaCl from this column and was >80% pure at this stage. After dialysis to remove salt the fusion protein could be cleaved by an enterokinase treatment as described in Example 1 to yield MIP-1α.

EXAMPLE 4 - THIOREDOXIN-BMP-2 FUSION MOLECULE

Human Bone Morphogenetic Protein 2 (BMP-2) was expressed at high levels in E. coli as a thioredoxin fusion protein using the modified expression vector described in Example 3. The DNA sequence encoding human IL-11 in the modified pALtrxA/EK/IL11$_{\Delta}$ Pro-581 (nucleotides 2599-3132) is replaced by the 345 nucleotide DNA sequence shown in Fig. 3 encoding full-length, mature human BMP-2 [Wozney et al, Science 242:1528-1534 (1988)].

In this case the thioredoxin-BMP-2 fusion protein appeared in the insoluble cellular fraction when strain GI724 containing the expression vector was grown in medium containing tryptophan at 37°C. However, when the temperature of the growth medium was lowered to 20°C the fusion protein was found in the soluble cellular fraction.

EXAMPLE 5 - THIOREDOXIN-SMALL PEPTIDE FUSION MOLECULES

Native E. coli thioredoxin was expressed at high levels in E. coli using strain GI724 containing the same plasmid expression vector described in Example 3 deleted for nucleotides 2569-3129, and employing the growth and induction protocol outlined in Example 1. Under these conditions thioredoxin accumulated to approximately 10% of the total protein, all of it in the soluble cellular fraction.

Fig. 4 illustrates insertion of 13 amino acid residues encoding an enterokinase cleavage site into the active site loop of thioredoxin, between residues G_{34} and P_{35} of the thioredoxin protein sequence. The fusion protein containing this internal enterokinase site was expressed at levels equivalent to native thioredoxin, and was cleaved with an enterokinase treatment as outlined in Example 1 above. The fusion protein was found to be as stable as native thioredoxin to heat treatments, being resistant to a 10 minute incubation at 80°C as described in Example 4.

Below ar listed twelve additional peptide insertions which

were also made into the active site loop of thioredoxin between G_{34} and P_{35}. The sequences are each 14 amino acid residues in length and are random in composition. Each of the thioredoxin fusion proteins containing these random insertions were made at levels comparable to native thioredoxin. All of them were found in the soluble cellular fraction. These peptides include the following sequences:

Pro-Leu-Gln-Arg-Ile-Pro-Pro-Gln-Ala-Leu-Arg-Val-Glu-Gly,
Pro-Arg-Asp-Cys-Val-Gln-Arg-Gly-Lys-Ser-Leu-Ser-Leu-Gly,
Pro-Met-Arg-His-Asp-Val-Arg-Cys-Val-Leu-His-Gly-Thr-Gly,
Pro-Gly-Val-Arg-Leu-Pro-Ile-Cys-Tyr-Asp-Asp-Ile-Arg-Gly,
Pro-Lys-Phe-Ser-Asp-Gly-Ala-Gln-Gly-Leu-Gly-Ala-Val-Gly,
Pro-Pro-Ser-Leu-Val-Gln-Asp-Asp-Ser-Phe-Glu-Asp-Arg-Gly,
Pro-Trp-Ile-Asn-Gly-Ala-Thr-Pro-Val-Lys-Ser-Ser-Ser-Gly,
Pro-Ala-His-Arg-Phe-Arg-Gly-Gly-Ser-Pro-Ala-Ile-Phe-Gly,
Pro-Ile-Met-Gly-Ala-Ser-His-Gly-Glu-Arg-Gly-Pro-Glu-Gly,
Pro-Asp-Ser-Leu-Arg-Arg-Arg-Glu-Gly-Phe-Gly-Leu-Leu-Gly,
Pro-Ser-Glu-Tyr-Pro-Gly-Leu-Ala-Thr-Gly-His-His-Val-Gly,
and Pro-Leu-Gly-Val-Leu-Gly-Ser-Ile-Trp-Leu-Glu-Arg-Gln-Gly.

The inserted sequences contained examples that were both hydrophobic and hydrophilic, and examples that contained cysteine residues. It appears that the active-site loop of thioredoxin can tolerate a wide variety of peptide insertions resulting in soluble fusion proteins. Standard procedures can be used to purify these loop "inserts".

EXAMPLE 6 - HUMAN INTERLEUKIN-6

Human interleukin-6 (IL-6) was expressed at high levels in *E. coli* as a thioredoxin fusion protein using an expression vector similar to modified pALtrxA/EK/IL11$_\Delta$ Pro-581 described in Example 3 above. To express a thioredoxin-IL-6 fusion the DNA sequence in modified pALtrxA/EK/IL11$_\Delta$ Pro-581 encoding human IL-11 (nucleotides 2599-3132) is replaced by the 561 nucleotide DNA sequenc shown in Figur 6 encoding full-length, mature human IL-6 [Hiran et al, *Nature* 324:73-76 (1986)]. Th host strain and

expression protocol used for the production of thioredoxin-IL-6 fusion protein are as described in Example 1.

When the fusion protein was synthesized at 37°C, approximately 50% of it was found in the "inclusion body" or insoluble fraction. However all of the thioredoxin-IL-6 fusion protein, representing up to 10% of the total cellular protein, was found in the soluble fraction when the temperature of synthesis was lowered to 25°C.

EXAMPLE 7 - HUMAN MACROPHAGE COLONY STIMULATING FACTOR

Human Macrophage Colony Stimulating Factor (M-CSF) was expressed at high levels in E. coli as a thioredoxin fusion protein using the modified expression vector similar to pALtrxA/EK/IL11$_{\Delta}$ Pro-581 described in Example 3 above.

The DNA sequence encoding human IL-11 in modified pALtrxA/EK/IL11$_{\Delta}$ Pro-581 (nucleotides 2599-3135) is replaced by the 669 nucleotide DNA sequence shown in Fig. 7 encoding the first 223 amino acids of mature human M-CSFβ [G. G. Wong et al, Science 235:1504-1508 (1987)]. The host strain and expression protocol used for the production of thioredoxin-M-CSF fusion protein was as described in Example 2 above.

As was seen with the thioredoxin-IL-11 fusion protein, all of the thioredoxin-M-CSF fusion protein was found in the soluble cellular fraction, representing up to 10% of the total protein.

EXAMPLE 8 - RELEASE OF FUSION PROTEIN VIA OSMOTIC SHOCK OR FREEZE/THAW

To determine whether the fusions of heterologous proteins to thioredoxin according to this invention enable targeting to the host cell's adhesion sites and permit the release of the fusion proteins from the cell, the cells were exposed to simple osmotic shock and freeze/thaw procedures.

Cells overproducing wild-type E. coli thioredoxin, human thi redoxin, th E. coli thior doxin-MIP1α fusion or the E. coli thi r doxin-IL-11 fusion were us d in the following procedur s.

For an osmotic shock treatment, cells were resuspended at 2 A_{550}/ml in 20 mM Tris-Cl pH 8.0/2.5 mM EDTA/20% w/v sucrose and kept cold on ice for 10 minutes. The cells were then pelleted by centrifugation (12,000 xg, 30 seconds) and gently resuspended in the same buffer as above but with sucrose omitted. After an additional 10 minute period on ice, to allow for the osmotic release of proteins, cells were re-pelleted by centrifugation (12,000 xg, 2 minutes) and the supernatant ("shockate") examined for its protein content. Wild-type E. coli thioredoxin and human thioredoxin were quantitatively released, giving "shockate" preparations which were >80% pure thioredoxin. More significantly >80% of the thioredoxin-MIP1α and >50% of the thioredoxin-IL-11 fusion proteins were released by this osmotic treatment.

A simple freeze/thaw procedure produced similar results, releasing thioredoxin fusion proteins selectively, while leaving most of the other cellular proteins inside the cell. A typical freeze/thaw procedure entails resuspending cells at 2 A_{550}/ml in 20 mM Tris-Cl pH 8.0/2.5 mM EDTA and quickly freezing the suspension in dry ice or liquid nitrogen. The frozen suspension is then allowed to slowly thaw before spinning out the cells (12,000 xg, 2 minutes) and examining the supernatant for protein.

Although the resultant "shockate" may require additional purification, the initial "shockate" is characterized by the absence of nucleic acid contaminants. Compared to an initial lysate, the purity of the "shockate" is significantly better, and does not require the difficult removal of DNA from bacterial lysates.

Thus, this release step can be substituted for the lysis step of Example 2. The supernatant obtained after centrifugation is then further purified in the manner disclosed in that Example.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expect d to b obvi us to n of skill in th art. Such modifications and alterations to the compositions and processes

of the present invention are believed to be encompassed in the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A DNA sequence encoding a fusion protein, said sequence comprising DNA encoding a thioredoxin-like protein fused to a DNA sequence encoding a selected heterologous protein.

2. A DNA sequence of claim 1 wherein said DNA encoding said thioredoxin-like protein comprises the amino terminus of said fusion protein.

3. A DNA sequence of claim 1 wherein said DNA encoding said thioredoxin-like protein comprises the carboxyl terminus of said fusion protein.

4. A DNA sequence of claim 1, 2 or 3 wherein said DNA encoding said thioredoxin-like protein is selected from the group consisting of E. coli thioredoxin and human thioredoxin.

5. A DNA sequence of claim 1, 2 or 3 wherein said DNA encoding said selected protein is selected from the group consisting of IL-11, IL-6, Macrophage Inhibitory Protein 1α and Bone Morphogenic Protein 2.

6. A DNA sequence of claim 1, 2 or 3 additionally comprising a linker DNA sequence fused between said DNA encoding said thioredoxin-like protein and said DNA encoding said selected heterologous protein.

7. A plasmid DNA molecule comprising a DNA sequence of claims 1-6, said sequence being under the control of a suitable expression control sequence capable of directing the expression of a fusion protein in a selected host cell.

8. An E. coli host cell transformed with, or having integrated into the genome thereof, a plasmid of claim 7.

9. A method of making a selected heterologous protein comprising

(a) culturing in a culture medium under suitable conditions a host cell of claim 8;

(b) recovering the fusion protein produced thereby from said culture medium;

(c) cleaving said selected heterologous protein from said fusion protein and

(d) isolating said selected heterologous protein.

10. IL-11 protein produced by the method of claim 9.

11. Use of thioredoxin in the method of claim 9.

FIGURE 1A

pALtrxA/EK/IL1a Pro-581

```
GACGAAAGGG CCTCGTGATA CGCCTATTTT TATAGGTTAA      40
TGTCATGATA ATAATGGTTT CTTAGACGTC AGGTGGCACT      80
TTTCGGGGAA ATGTGCGCGG AACCCCTATT TGTTTATTTT     120
TCTAAATACA TTCAAATATG TATCCGCTCA TGAGACAATA     160
ACCCTGATAA ATGCTTCAAT AATATTGAAA AAGGAAGAGT     200
ATGAGTATTC AACATTTCCG TGTCGCCCTT ATTCCCTTTT     240
TTGCGGCATT TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC     280
GCTGGTGAAA GTAAAAGATG CTGAAGATCA GTTGGGTGCA     320
CGAGTGGGTT ACATCGAACT GGATCTCAAC AGCGGTAAGA     360
TCCTTGAGAG TTTTCGCCCC GAAGAACGTT TTCCAATGAT     400
GAGCACTTTT AAAGTTCTGC TATGTGGCGC GGTATTATCC     440
CGTATTGACG CCGGGCAAGA GCAACTCGGT CGCCGCATAC     480
ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC     520
AGAAAAGCAT CTTACGGATG GCATGACAGT AAGAGAATTA     560
TGCAGTGCTG CCATAACCAT GAGTGATAAC ACTGCGGCCA     600
ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC     640
CGCTTTTTTG CACAACATGG GGGATCATGT AACTCGCCTT     680
GATCGTTGGG AACCGGAGCT GAATGAAGCC ATACCAAACG     720
ACGAGCGTGA CACCACGATG CCTGTAGCAA TGGCAACAAC     760
GTTGCGCAAA CTATTAACTG GCGAACTACT TACTCTAGCT     800
TCCCGGCAAC AATTAATAGA CTGGATGGAG GCGGATAAAG     840
TTGCAGGACC ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG     880
GTTTATTGCT GATAAATCTG GAGCCGGTGA GCGTGGGTCT     920
CGCGGTATCA TTGCAGCACT GGGGCCAGAT GGTAAGCCCT     960
CCCGTATCGT AGTTATCTAC ACGACGGGGA GTCAGGCAAC    1000
TATGGATGAA CGAAATAGAC AGATCGCTGA GATAGGTGCC    1040
```

FIGURE 1B

TCACTGATTA AGCATTGGTA ACTGTCAGAC CAAGTTTACT	1080
CATATATACT TTAGATTGAT TTAAAACTTC ATTTTTAATT	1120
TAAAAGGATC TAGGTGAAGA TCCTTTTTGA TAATCTCATG	1160
ACCAAAATCC CTTAACGTGA GTTTTCGTTC CACTGAGCGT	1200
CAGACCCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC	1240
TTTTTTTCTG CGCGTAATCT GCTGCTTGCA AACAAAAAAA	1280
CCACCGCTAC CAGCGGTGGT TTGTTTGCCG GATCAAGAGC	1320
TACCAACTCT TTTTCCGAAG GTAACTGGCT TCAGCAGAGC	1360
GCAGATACCA AATACTGTCC TTCTAGTGTA GCCGTAGTTA	1400
GGCCACCACT TCAAGAACTC TGTAGCACCG CCTACATACC	1440
TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG	1480
CGATAAGTCG TGTCTTACCG GGTTGGACTC AAGACGATAG	1520
TTACCGGATA AGGCGCAGCG GTCGGGCTGA ACGGGGGGTT	1560
CGTGCACACA GCCCAGCTTG GAGCGAACGA CCTACACCGA	1600
ACTGAGATAC CTACAGCGTG AGCATTGAGA AAGCGCCACG	1640
CTTCCCGAAG GGAGAAAGGC GGACAGGTAT CCGGTAAGCG	1680
GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG	1720
GGGAAACGCC TGGTATCTTT ATAGTCCTGT CGGGTTTCGC	1760
CACCTCTGAC TTGAGCGTCG ATTTTTGTGA TGCTCGTCAG	1800
GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCTT	1840
TTTACGGTTC CTGGCCTTTT GCTGGCCTTT TGCTCACATG	1880
TTCTTTCCTG CGTTATCCCC TGATTCTGTG GATAACCGTA	1920
TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG	1960
AACGACCGAG CGCAGCGAGT CAGTGAGCGA GGAAGCGGAA	2000
GAGCGCCCAA TACGCAAACC GCCTCTCCCC GCGCGTTGGC	2040
CGATTCATTA ATGCAGAATT GATCTCTCAC CTACCAAACA	2080
ATGCCCCCCT GCAAAAAATA AATTCATATA AAAAACATAC	2120

FIGURE 1C

```
AGATAACCAT CTGCGGTGAT AAATTATCTC TGGCGGTGTT                    2160

GACATAAATA CCACTGGCGG TGATACTGAG CACATCAGCA                    2200

GGACGCACTG ACCACCATGA ATTCAAGAAG GAGATATACA                    2240

T ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC GAC                  2274
  Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp
    1               5                  10

AGT TTT GAC ACG GAT GTA CTC AAA GCG GAC GGG                    2307
Ser Phe Asp Thr Asp Val Leu Lys Ala Asp Gly
               15                  20

GCG ATC CTC GTC GAT TTC TGG GCA GAG TGG TGC                    2340
Ala Ile Leu Val Asp Phe Trp Ala Glu Trp Cys
          25                  30

GGT CCG TGC AAA ATG ATC GCC CCG ATT CTG GAT                    2373
Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp
     35                  40

GAA ATC GCT GAC GAA TAT CAG GGC AAA CTG ACC                    2406
Glu Ile Ala Asp Glu Tyr Gln Gly Lys Leu Thr
 45                  50                  55

GTT GCA AAA CTG AAC ATC GAT CAA AAC CCT GGC                    2439
Val Ala Lys Leu Asn Ile Asp Gln Asn Pro Gly
               60                  65

ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG                    2472
Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro
          70                  75

ACT CTG CTG CTG TTC AAA AAC GGT GAA GTG GCG                    2505
Thr Leu Leu Leu Phe Lys Asn Gly Glu Val Ala
     80                  85

GCA ACC AAA GTG GGT GCA CTG TCT AAA GGT CAG                    2538
Ala Thr Lys Val Gly Ala Leu Ser Lys Gly Gln
 90                  95

TTG AAA GAG TTC CTC GAC GCT AAC CTG GCC GGT                    2571
Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala Gly
100                 105                 110

TCT GGT TCT GGT GAT GAC GAT GAC AAA GGT CCA                    2604
Ser Gly Ser Gly Asp Asp Asp Asp Lys Gly Pro
               115                 120
```

FIGURE 1D

```
CCA CCA GGT CCA CCT CGA GTT TCC CCA GAC CCT                    2637
Pro Pro Gly Pro Pro Arg Val Ser Pro Asp Pro
            125                 130

CGG GCC GAG CTG GAC AGC ACC GTG CTC CTG ACC                    2670
Arg Ala Glu Leu Asp Ser Thr Val Leu Leu Thr
        135                 140

CGC TCT CTC CTG GCG GAC ACG CGG CAG CTG GCT                    2703
Arg Ser Leu Leu Ala Asp Thr Arg Gln Leu Ala
    145                 150

GCA CAG CTG AGG GAC AAA TTC CCA GCT GAC GGG                    2736
Ala Gln Leu Arg Asp Lys Phe Pro Ala Asp Gly
155                 160                 165

GAC CAC AAC CTG GAT TCC CTG CCC ACC CTG GCC                    2769
Asp His Asn Leu Asp Ser Leu Pro Thr Leu Ala
                170                 175

ATG AGT GCG GGG GCA CTG GGA GCT CTA CAG CTC                    2802
Met Ser Ala Gly Ala Leu Gly Ala Leu Gln Leu
            180                 185

CCA GGT GTG CTG ACA AGG CTG CGA GCG GAC CTA                    2835
Pro Gly Val Leu Thr Arg Leu Arg Ala Asp Leu
        190                 195

CTG TCC TAC CTG CGG CAC GTG CAG TGG CTG CGC                    2868
Leu Ser Tyr Leu Arg His Val Gln Trp Leu Arg
    200                 205

CGG GCA GGT GGC TCT TCC CTG AAG ACC CTG GAG                    2901
Arg Ala Gly Gly Ser Ser Leu Lys Thr Leu Glu
210                 215                 220

CCC GAG CTG GGC ACC CTG CAG GCC CGA CTG GAC                    2934
Pro Glu Leu Gly Thr Leu Gln Ala Arg Leu Asp
            225                 230

CGG CTG CTG CGC CGG CTG CAG CTC CTG ATG TCC                    2967
Arg Leu Leu Arg Arg Leu Gln Leu Leu Met Ser
        235                 240

CGC CTG GCC CTG CCC CAG CCA CCC CCG GAC CCG                    3000
Arg Leu Ala Leu Pro Gln Pro Pro Pro Asp Pro
    245                 250

CCG GCG CCC CCG CTG GCG CCC CCC TCC TCA GCC                    3033
Pro Ala Pro Pro Leu Ala Pro Pro Ser Ser Ala
255                 260
```

FIGURE 1E

```
TGG GGG GGC ATC AGG GCC GCC CAC GCC ATC CTG                    3066
Trp Gly Gly Ile Arg Ala Ala His Ala Ile Leu
265                 270                 275

GGG GGG CTG CAC CTG ACA CTT GAC TGG GCC GTG                    3099
Gly GLy Leu His Leu Thr Leu Asp Trp Ala Val
                280                 285

AGG GGA CTG CTG CTG CTG AAG ACT CGG CTG TGA                    3132
Arg Gly Leu Leu Leu Leu Lys Thr Arg Leu
            290                 295

AAGCTTATCG ATACCGTCGA CCTGCAGTAA TCGTACAGGG                    3172

TAGTACAAAT AAAAAAGGCA CGTCAGATGA CGTGCCTTTT                    3212

TTCTTGTGAG CAGTAAGCTT GGCACTGGCC GTCGTTTTAC                    3252

AACGTCGTGA CTGGGAAAAC CCTGGCGTTA CCCAACTTAA                    3292

TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT                    3332

AGCGAAGAGG CCCGCACCGA TCGCCCTTCC CAACAGTTGC                    3372

GCAGCCTGAA TGGCGAATGG CGCCTGATGC GGTATTTTCT                    3412

CCTTACGCAT CTGTGCGGTA TTTCACACCG CATATATGGT                    3452

GCACTCTCAG TACAATCTGC TCTGATGCCG CATAGTTAAG                    3492

CCAGCCCCGA CACCCGCCAA CACCCGCTGA CGCGCCCTGA                    3532

CGGGCTTGTC TGCTCCCGGC ATCCGCTTAC AGACAAGCTG                    3572

TGACCGTCTC CGGGAGCTGC ATGTGTCAGA GGTTTTCACC                    3612

GTCATCACCG AAACGCGCGA                                         3632
```

FIGURE 2

MIP-1α

```
GCA CCA CTT GCT GCT GAC ACG CCG ACC GCC TGC TGC     36
Ala Pro Leu Ala Ala Asp Thr Pro Thr Ala Cys Cys
  1               5                  10

TTC AGC TAC ACC TCC CGA CAG ATT CCA CAG AAT TTC     72
Phe Ser Tyr Thr Ser Arg Gln Ile Pro Gln Asn Phe
          15                  20

ATA GCT GAC TAC TTT GAG ACG AGC AGC CAG TGC TCC    109
Ile Ala Asp Tyr Phe Glu Thr Ser Ser Gln Cys Ser
 25                  30                  35

AAG CCC AGT GTC ATC TTC CTA ACC AAG AGA GGC CGG    145
Lys Pro Ser Val Ile Phe Leu Thr Lys Arg Gly Arg
              40                  45

CAG GTC TGT GCT GAC CCC AGT GAG GAG TGG GTC CAG    181
Gln Val Cys Ala Asp Pro Ser Glu Glu Trp Val Gln
     50                  55                  60

AAA TAC GTC AGT GAC CTG GAG CTG AGT GCC TAA        214
Lys Thr Val Ser Asp Leu Glu Leu Ser Ala
                 65                  70
```

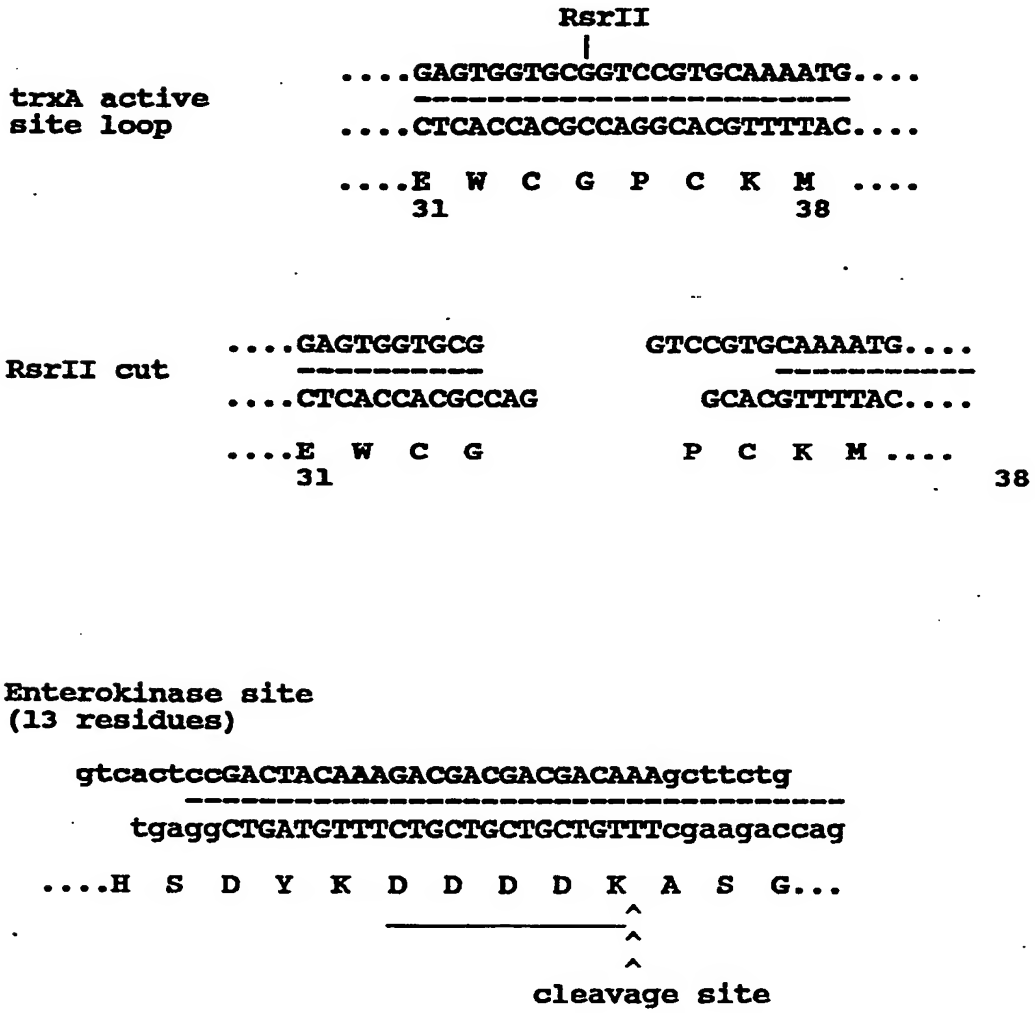
FIGURE 3

BMP-2

```
CAA GCT AAA CAT AAA CAA CGT AAA CGT CTG AAA TCT     36
Gln Ala Lys His Lys Gln Arg Lys Arg Leu Lys Ser
  1               5                  10

AGC TGT AAG AGA CAC CCT TTG TAC GTG GAC TTC AGT     72
Ser Cys Lys Arg His Pro Leu Tyr Val Asp Phe Ser
         15                  20

GAC GTG GGG TGG AAT GAC TGG ATT GTG GCT CCC CCG    109
Asp Val Gly Trp Asn Asp Trp Ile Val Ala Pro Pro
 25                  30                  35

GGG TAT CAC GCC TTT TAC TGC CAC GGA GAA TGC CCT    145
Gly Tyr His Ala Phe Tyr Cys His Gly Glu Cys Pro
             40                  45

TTT CCT CTG GCT GAT CAT CTG AAC TCC ACT AAT CAT    181
Phe Pro Leu Ala Asp His Leu Asn Ser Thr Asn His
     50                  55                  60

GCC ATT GTT CAG ACG TTG GTC AAC TCT GTT AAC TCT    217
Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser
                 65                  70

AAG ATT CCT AAG GCA TGC TGT GTC CCG ACA GAA CTC    253
Lys Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu
         75                  80

AGT GCT ATC TCG ATG CTG TAC CTT GAC GAG AAT GAA    289
Ser Ala Ile Ser Met Leu Tyr Leu Asp Glu Asn Glu
 85                  90                  95

AAG GTT GTA TTA AAG AAC TAT CAG GAC ATG GTT GTG    325
Lys Val Val Leu Lys Asn Tyr Gln Asp Met Val Val
             100                 105

GAG GGT TGT GGG TGT CGC TAG                       346
Glu Gly Cys Gly Cys Arg
    110
```

FIGURE 4

INSERTION OF AN ENTEROKINASE SITE INTO
THE ACTIVE-SITE LOOP OF E.COLI THIOREDOXIN (trxA)



Enterokinase site
(13 residues)

FIGURE 5

RANDOM PEPTIDE INSERTIONS INTO THE ACTIVE-SITE LOOP OF E.COLI THIOREDOXIN (trxA)

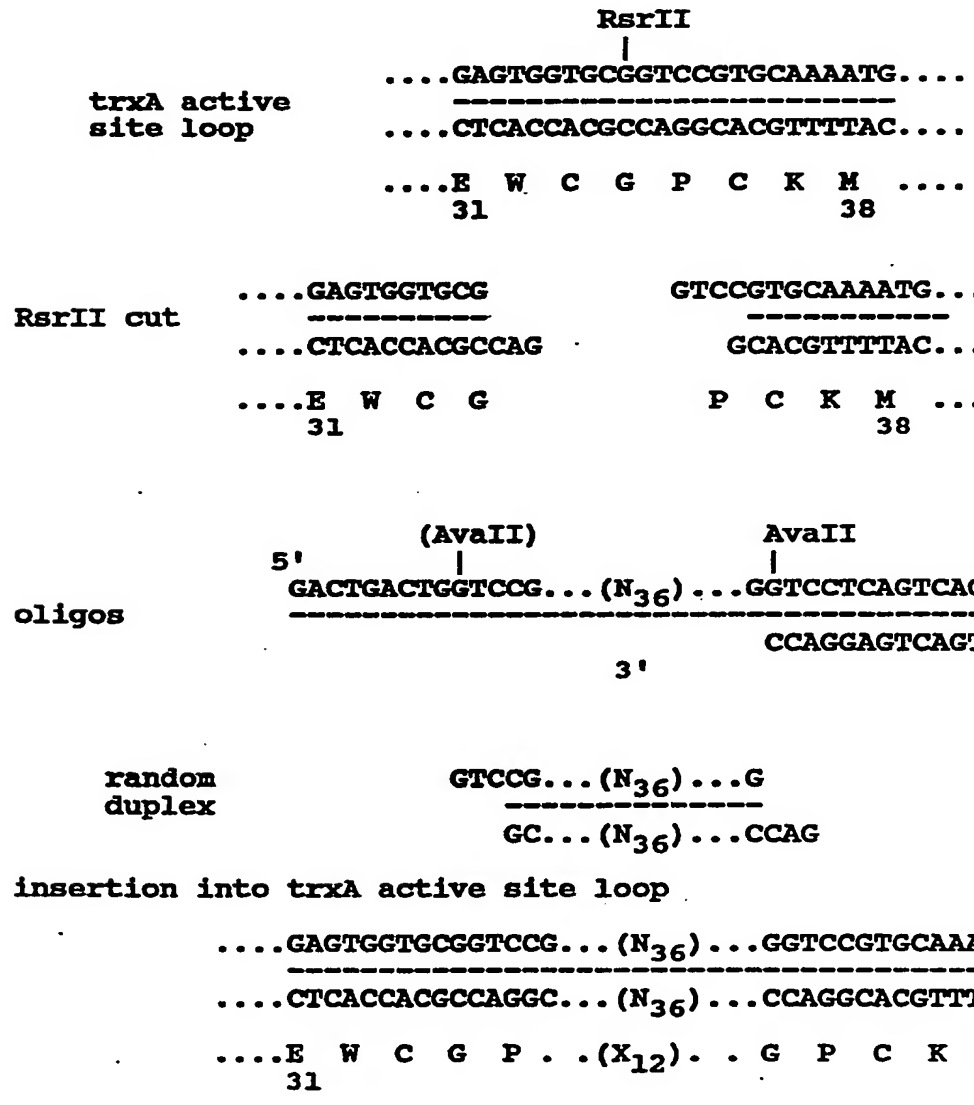


FIGURE 6

IL-6

```
                        5                  10
ATG GCT CCA GTA CCT CCA GGT GAA GAT TCT AAA GAT GTA   39
Met Ala Pro Val Pro Pro Gly Glu Asp Ser Lys Asp Val

     15                 20                  25
GCC GCC CCA CAC AGA CAG CCA CTC ACC TCT TCA GAA CGA   78
Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg

                   30                  35
ATT GAC AAA CAA ATT CGG TAC ATC CTC GAC GGC ATC TCA  117
Ile Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser

     40                 45                  50
GCC CTG AGA AAG GAG ACA TGT AAC AAG AGT AAC ATG TGT  156
Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser Asn Met Cys

               55                  60
GAA AGC AGC AAA GAG GCA CTG GCA GAA AAC AAC CTG AAC  195
Glu Ser Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn

 65                 70                  75
CTT CCA AAG ATG GCT GAA AAA GAT GGA TGC TTC CAA TCT  234
Leu Pro Lys Met Ala Glu Lys Asp Gly Cys Phe Gln Ser

         80                  85                  90
GGA TTC AAT GAG GAG ACT TGC CTG GTG AAA ATC ATC ACT  273
Gly Phe Asn Glu Glu Thr Cys Leu Val Lys Ile Ile Thr

                   95                 100
GGT CTT TTG GAG TTT GAG GTA TAC CTA GAG TAC CTC CAG  312
Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Thr Leu Gln

         105                 110                 115
AAC AGA TTT GAG AGT AGT GAG GAA CAA GCC AGA GCT GTG  351
Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val

                   120                 125
CAG ATG AGT ACA AAA GTC CTG ATC CAG TTC CTG CAG AAA  390
Gln Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln Lys
```

FIGURE 6 (continued)

```
    130                 140                 150
AAG GCA AAG AAT CTA GAT GCA ATA ACC ACC CCT GAC CCA  429
Lys Ala Lys Asn Leu Asp Ala Ile Thr Thr Pro Asp Pro

            155                 160
ACC ACA AAT GCC AGC CTG CTG ACG AAG CTG CAG GCA CAG  468
Thr Thr Asn Ala Ser Leu Leu Thr Lys Leu Gln Ala Gln

    170                 175                 180
AAC CAG TGG CTG CAG GAC ATG ACA ACT CAT CTC ATT CTG  507
Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile Leu

            185                 190
CGC AGC TTT AAG GAG TTC CTG CAG TCC AGC CTG AGG GCT  546
Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala

195
CTT CGG CAA ATG TAG                                 561
Leu Arg Gln Met  *
```

FIGURE 7

```
   1 GAAGAAGTTT CTGAATATTG TAGCCACATG ATTGGGAGTG GACACCTGCA
  51 GTCTCTGCAG CGGCTGATTG ACAGTCAGAT GGAGACCTCG TGCCAAATTA
 101 CATTTGAGTT TGTAGACCAG GAACAGTTGA AAGATCCAGT GTGCTACCTT
 151 AAGAAGGCAT TTCTCCTGGT ACAAGACATA ATGGAGGACA CCATGCGCTT
 201 CAGAGATAAC ACCCCCAATG CCATCGCCAT TGTGCAGCTG CAGGAACTCT
 251 CTTTGAGGCT GAAGAGCTGC TTCACCAAGG ATTATGAAGA GCATGACAAG
 301 GCCTGCGTCC GAACTTTCTA TGAGACACCT CTCCAGTTGC TGGAGAAGGT
 351 CAAGAATGTC TTTAATGAAA CAAAGAATCT CCTTGACAAG GACTGGAATA
 401 TTTTCAGCAA GAACTGCAAC AACAGCTTTG CTGAATGCTC CAGCCAAGAT
 451 GTGGTGACCA AGCCTGATTG CAACTGCCTG TACCCCAAAG CCATCCCTAG
 501 CAGTGACCCG GCCTCTGTCT CCCCTCATCA GCCCCTCGCC CCCTCCATGG
 551 CCCCTGTGGC TGGCTTGACC TGGGAGGACT CTGAGGGAAC TGAGGGCAGC
 601 TCCCTCTTGC CTGGTGAGCA GCCCCTGCAC ACAGTGGATC CAGGCAGTGC
 651 CAAGCAGCGG CCACCCAGG
```

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00944

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C 12N 15/70, 1/21, C07H 21/04; C07K 13/00; C12P 21/02
US CL : 435/69.1, 252.33, 320.1; 530/351; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/69.1, 69.7, 240.1, 243, 252.33, 320.1; 530/351; 536/27; 935/10, 27, 72

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X/Y	Proceedings of the National Academy of Sciences, U.S.A. Volume 87, issued November 1990, N. Wakasugi et al. "Adult T-cell leukemia-derived factor/thioredoxin, produced by both human T-lymphotrophic virus tyoe I- and Epstein-Barr virus-transformed lymphocytes, acts as an autocrine growth factor and synergizes with interleukin 1 and interleukin 2", pages 8282-8286, see introduction.	1-4, 6-9 / 5, 11
X/Y,P	US,A, 5,011,772 (Recsei) 30 April 1991, see Example 4.	1-4, 6-9/ 5,11
X/Y	Proceedings of the National Academy of Sciences, U.S.A., Volume 87, issued October 1990, S.R. Paul et al, "Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine", pages 7512-7516, see entire document.	9/5

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
22 APRIL 1992	13 MAY 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Gabriele E. Bugaisky

Form PCT/ISA/210 (second sheet)(May 1986) 8

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-9 and 11, drawn to a first composition and a fisrt method of use, classified in Class 435/69.1.
II. Claim 10, drawn to a second composition, classified in Class 530/351.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

II. FIELDS SEARCHED
Other Documents Searched:

APS; DIALOG: MEDLINE, BIOSIS, BIOTECH ABS; GENBANK, PIR. search terms: interleukin 6, interleukin 11, IL-6, IL-11, fusion protein, fusion peptide, thioredoxin, chimeric protein, prrotein, chimera, macrophage inhibitory protein 1α, MIP-1α, bone morphogenic protein 2, BMP 2, interleukin 1, IL-1, glutaredoxin, protein disulfide isomerase, stabilization, stabilize, stability, accumulation, accumulate